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Since viral oncoproteins are expected to compete with and imitate interactions that pRB has with cyclin D1, understanding high affinity pRB-viral oncoprotein complexes will provide tremendous insight into the specific interactions required for the development of small compounds that can destabilize pRB-cyclin D1 complexes in cyclin D1 mediated breast cancer. A structure-based approach is used to understand the molecular mechanisms that are necessary for high affinity pRB-viral oncoprotein interaction. The recently determined pRB-E7 peptide crystal structure[31] has significant shortcomings because the crystallized E7 nine amino acid peptide binds to pRB with a twenty-fold weaker affinity compared to full-length HPV16 E7[32]. Therefore, the goal of this project is to determine the three dimensional structure of pRB bound to larger regions of HPV E7, and Adenovirus 5 E1A.

This study demonstrated that bacterially coexpressed pRB(376-792) and viral oncoproteins formed complexes. These purified complexes probably have resisted crystallization because pRB(376-792) included a flexible linker region between two structured domains of pRB. Partial proteolysis of pRB-viral oncoprotein complexes yielded three protein fragments that are consistent with the molecular weights of pRB structured domains and larger viral oncoprotein regions (which contain high affinity pRB binding domains). Therefore, we expect that limited proteolysis will result in crystallizable pRB-viral oncoprotein complexes.

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Adrienne Clements 6/24/98

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INTRODUCTION:

BACKGROUND

pRB in Cancer

There are two general classes of genes that are associated with the development of tumors. The first of these classes is composed of oncogenes, which encode proteins capable of transforming normal cells into cancerous cells [1]. The second class of genes encode proteins that suppress tumor formation. Deletions or mutations of tumor suppressor genes are found in a variety of cancer types [2].

One of the first tumor suppressor genes identified was the retinoblastoma susceptibility gene (RB) [3, 4]. Mutational inactivation of the RB gene was originally correlated with the pediatric disease retinoblastoma [3,4,5]. More recently, it was demonstrated that structural abnormalities of the RB gene are associated with a wide variety of other tumors, including osteosarcoma, soft tissue sarcomas, and lung and breast carcinomas [reviewed in 6]. Loss of heterozygosity of the RB gene on chromosomal position 13q14 is a common occurrence in these tumors [6].

The association of RB gene mutations with breast cancer is particularly striking. Specifically, it was reported that allele loss occurred on chromosomal region 13q in 40% of human primary breast carcinomas tested [7]. Additionally, in a significant number of examined human breast tumor samples, the RB gene contained deletions or internal duplications that lead to either a complete loss or truncation of RB transcripts [reviewed in 8, 9]. T'ang *et al.* reported that as many as 25% of the breast cancer cell lines tested contained deletions or rearrangements of the RB gene [10]. Moreover, Varley, *et al.* found that 19% of primary breast carcinomas also contained structural abnormalities in the RB gene [11]. These mutations lead to deficient pRB expression in mammary tumors. Immunohistochemical methods demonstrated that 29% of examined breast tumors contained a proportion of cells that had undetectable levels of the retinoblastoma tumor suppressor protein (pRB) [11]. This suggests that pRB expression is required for normal cell division.

pRB in Cell Cycle

The RB gene encodes a 928 amino acid nuclear phosphoprotein that is essential for proper regulation of cell division [12]. Cell division is regulated by a process called the cell cycle. The transitions between various stages of the cell cycle are controlled by a series of checkpoints. These checkpoints govern the initiation and the completion of DNA replication (S phase), cell division (M phase), and the two gaps separating these phases, G1 and G2 [13]. The checkpoint that allows the cell to progress from G1 to S phase is referred to as the restriction point in mammalian cells. Once the cell has proceeded through this particular checkpoint, it has committed to undergo one round of DNA replication and can no longer respond to external stimuli [13]. pRB is required for proper regulation of the G1/S phase transition in the cell cycle [13] and oncogenesis is usually associated with deregulation of this restriction point. Proper regulation of pRB is mediated by the kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes [13].

The amount of pRB in all phases of the cell cycle is fairly constant. This protein is reversibly regulated during various stages of the cell cycle by alterations in its phosphorylation state [14]. pRB is hypophosphorylated in the G1 phase of the cell cycle and becomes increasingly phosphorylated as the cell cycle progresses through the G2/M phase transition [14]. In early G1, hypophosphorylated pRB normally exists as a complex with E2F transcription factors 1, 2, or 3. These transcription factors are involved in the stimulation of transcription of S-phase-specific genes [14]. In late G1, pRB is

phosphorylated by CDKs, which causes pRB to release E2F transcription factors [Figure 1]. This event allows for the transcription of genes that are involved in cellular proliferation [14]. pRB is then dephosphorylated by phosphatase type 1 during late M phase [12].

Relationship between pRB and Cyclin D1

Phosphorylation of pRB occurs at several serine and threonine residues of consensus cyclin-CDK phosphorylation sites [15]. The discovery that cyclin D expression was highly correlated with the cell cycle restriction point [12] suggested that the cyclin D- CDK4 complex was responsible for pRB phosphorylation during the G1 phase of the cell cycle. Reports of direct physical interaction between cyclin D1 and pRB support this observation [16-18]. Moreover, *in vitro* studies also demonstrate that pRB [18] and E2F1 [19] are substrates for cyclin D1-CDK4 complexes. Ectopically expressed cyclin D1 is associated with early pRB phosphorylation and early progression through G1 phase [20]. Moreover, microinjection of cyclin D1 antibodies cause most cell types to arrest before S phase [21]. This suggests that cyclin D1 is necessary for proper regulation of pRB and is involved in the G1/S phase transition.

The relationship between cyclin D1 and pRB seems to be interdependent. In addition to the proposed regulation of pRB by cyclin D1, it has been demonstrated that there is significantly less cyclin D1 and cyclin D1-CDK4 complexes in cells that lack functional pRB [22]. This indicates that the hypophosphorylated form of pRB may be involved in cyclin D1 expression. Additionally, Muller *et al.* demonstrated that exogenously expressed pRB is capable of inducing the expression of cyclin D1 [23].

In addition to its interaction with cyclin D and E2F transcription factors, pRB is also capable of binding to the DNA viral oncoproteins adenovirus E1A, simian virus 40 (SV40) large T antigen, and human papillomavirus (HPV)16 E7 [14]. Of the three DNA tumor viruses, only the high risk forms of HPV have been implicated in the genesis of cancer [12]. However, all of these oncoproteins are modular and share similar biological functions [12]. The most notable of these functions is that these proteins are able to bind to pRB in its hypophosphorylated state [12]. This interaction causes the hypophosphorylated pRB to release its associated E2F transcription factors prematurely [12, Figure 2]. Therefore, interaction between these viral oncoproteins and pRB contributes to the premature entry into the S phase of the cell cycle [14].

These viral oncoproteins, along with cyclin D and other cellular RB binding proteins, contain an LXCXE amino acid sequence (where X is any amino acid) that is necessary for binding to pRB [14, Figure 2]. The minimal binding region on pRB that is required for interaction with the DNA viral oncoproteins is referred to as the small pocket (residues 393-772). This small pocket consists of two domains. These domains are separated by a linker region that can be replaced by a random sequence of 12 amino acids [12]. The first domain of pRB consists of amino acids 393-572 and the second domain contains amino acids 646-772 [24]. It is interesting to note that all stable naturally occurring nonfunctional mutants of pRB contain mutations that map to this small pocket, suggesting that this region is necessary for cell cycle regulation [25,26]. Many of these mutants are also incapable of binding to the viral oncoproteins and are not phosphorylated [25,26]. A sequence that is C-terminal to this small pocket is also required for efficient phosphorylation [12] and E2F interaction [14]. The small pocket plus the C-terminal region is referred to as the large pocket of pRB [12]. This 60 kilodalton large pocket of pRB is sufficient for cell cycle regulation and for the association with E2F transcription factors [12].

In addition to the correlation between RB gene mutations and breast cancer, it was also demonstrated that cyclin D1 is overexpressed in a large number of mammary carcinomas that have normal pRB expression. Bartkova *et al.* reported that irregular nuclear overexpression and accumulation of the cyclin D1 protein occurred in approximately half of the 170 primary breast carcinomas samples

examined by immunohistochemistry [27]. This indicates that the frequency of cyclin D1 abnormalities in breast cancer is approximately 15-20% higher than DNA amplification studies have predicted [28]. Furthermore, when DNA tumor viral protein adenovirus E1A, SV40 large T antigen, or HPV16 E7 is present, cyclin D1 expression is down regulated [27]. Since these DNA tumor viral proteins bind to and inactivate hypophosphorylated pRB, this suggests that active G1 phase pRB is involved in cyclin D1 overexpression. Therefore, it appears that there is a tremendous amount of mutual dependence between pRB and cyclin D1 cell cycle regulators. Abnormalities in this pRB-cyclin D1 pathway seem to contribute significantly to the development of mammary carcinomas, as well as many other tumor types [27]. In summary, aberrations of this pathway in breast cancer involve RB gene mutations, cyclin D1 gene amplifications, and cyclin D1 overexpression that is not related to gene amplification [27].

PURPOSE/HYPOTHESIS

Since DNA viral oncoproteins and the cellular cyclin D protein share homologous regions that are essential for interaction with pRB, we hypothesize that these viral oncoproteins compete with and possibly imitate some interactions that pRB normally has with cyclin D and other cellular proteins. Studying how these viral proteins interact with pRB is expected to provide general insights into the interactions between pRB and cyclin D.

The HPV E7 proteins are the specific pRB-binding proteins that are useful for our studies. Unlike many cellular proteins, E7 proteins are small, well-behaved, and express easily in a bacterial system. Along with high risk HPV16 E7, human papillomaviral E7 proteins from other serotypes are capable of binding to pRB with different affinities [29]. Specifically, we are interested in the low risk HPV1a E7. This E7 protein contains the same amino acid sequence that is required for pRB interaction. However, HPV1a E7 binds to pRB with an affinity approximately 34% lower than the high risk HPV 16 E7 [29].

The cyclin D1/CDK4 complex has been demonstrated to phosphorylate pRB. Overexpressed cyclin D1 in breast cancer potentially deregulates the cell cycle by promoting aberrant pRB phosphorylation. Therefore, an understanding of pRB-protein interactions is essential to the rational design of drugs aimed at controlling cyclin D1-mediated breast cancer. In order to fully understand the mechanisms of pRB-protein interaction, it is necessary to determine the structure of biologically relevant pRB-protein complexes. Therefore, the primary goal of this research project is to determine the structures of the low risk HPV1a E7-pRB complex and the high risk HPV16 E7-pRB complex by utilizing X-ray crystallography. There are three reasons for attempting to determine the structure of pRB in these complexes. First, many proteins that are unable to crystallize alone due to protein structural instability crystallize easily when complexed with ligands, peptides, or inhibitors [reviewed in 30]. Supporting this idea, it has been demonstrated that an E7-derived peptide (amino acids 20-29) significantly increases the thermal stability of the pRB large pocket domain. Secondly, due to the similar sequences among the small E7 proteins and other cellular proteins that interact with pRB, these particular complexes are expected to provide significant understanding of how cyclin D1 and potentially other cellular proteins also interact with pRB. Finally, the comparison of these two E7-pRB complexes should demonstrate the differences between a low affinity pRB-binding region and a high affinity pRB-binding region. The molecular components that establish these differences will provide tremendous insight into the specific interactions required for the development of small compounds that can destabilize pRB-cyclin D1 complexes in cyclin D1-mediated breast cancer.

Recently, the three dimensional structure of the pRB small pocket domain bound to a nine amino acid HPV16 E7 peptide (amino acids 20 -29) was solved by X-ray crystallography[31]. This structure provides insight into pRB-LXCXE interaction. However, this crystallized HPV16 E7-derived peptide binds to pRB with a twenty-fold weaker affinity compared with full-length HPV16 E7[32] and is

incapable of displacing E2F transcription factors[33]. This suggests that a region of HPV16 E7 outside of amino acids 20-29 makes additional contacts with pRB. In support of this hypothesis, a study by Patrick, *et al.* demonstrates that a region C-terminal to the crystallized HPV16 E7 sequence contains an additional pRB binding site[33]. Therefore, in order to understand how viral oncoproteins compete with Cyclin D1 for pRB binding, the molecular mechanisms of high affinity pRB-viral oncoprotein interactions must be elucidated. In order to understand the molecular components involved in high affinity pRB-viral oncoprotein interaction, the constructs of HPV16 E7 and HPV1a E7 utilized in this project include the C-terminal pRB binding region.

TECHNICAL OBJECTIVES

The specific objectives for this project are: 1) to express and purify constructs of HPV 16 E7, HPV1a E7, and the small and large pocket domains of pRB, 2) to prepare a pRB-HPV16 E7 and a pRB-HPV1a E7 complex that is suitable for structure determination by X-ray crystallographic techniques, 3) to determine the three-dimensional structure of these pRB-E7 complexes by x-ray crystallography in order to study the essential molecular components for high affinity pRB-protein interactions.

BODY:

EXPERIMENTAL METHODS AND RESULTS

The cDNA of HPV16 E7 and HPV1a E7 was obtained from Dr. Robert Ricciardi and Dr. Thomas Ifter respectively. Two constructs of HPV16 E7 and of HPV 1a E7 were subcloned into a pRSETA vector for protein expression with a T7 promoter-T7 polymerase expression system in the bacterial strain BL21(DE3). Constructs of the full length HPV16 E7 (amino acids 1-98) and full length HPV1a E7 (amino acids 1-93) were produced and include three highly conserved regions (CR1-CR3, Figure 3a) among DNA viral oncoproteins HPV E7, Adenovirus E1A, SV40 Large T antigen. Constructs containing the minimal pRB binding domains (CR2-CR3, Figure 3a) were also generated for HPV16 E7 (amino acids 17-98) and HPV1a E7 (amino acids 16-93). These constructs were made because the CR2 region of E7 contains the minimal pRB binding region (the LXCXE amino acid sequence)[14] and the CR3 region contains a second pRB binding site[33].

All HPV16 E7 and HPV1a E7 constructs were soluble when expressed for three hours at 37°C in BL21(DE3). Full length HPV16 E7, full length HPV1a E7, and HPV16 E7 (17-98) were purified to homogeneity in nondenaturing conditions. The purification schemes for these proteins consisted of a combination of anion exchange (Q-sepharose), hydrophobic interaction (phenyl-sepharose), and size exclusion chromatography on a Superdex-200 FPLC column. Each of the HPV E7 proteins eluted from the gel filtration column in one peak. A comparison to gel filtration protein standards indicated that the size of these proteins were larger than 44 kDa and smaller than 158 kDa (monomer molecular weight=11.022 kDa). However, subsequent analysis of purified HPV16 E7 constructs by sedimentation equilibrium indicated that these proteins were actually dimers.

In addition to the E7 constructs, comparable constructs of the highly homologous Adenovirus 5 E1A containing CR1-CR3 (amino acids 36-189) and CR2-CR3 (amino acids 114-189) were subcloned into pRSETA for bacterial expression (Figure 3b). The cDNA for Adenovirus 5 E1A was obtained from Dr. Robert Ricciardi. E1A was included in this study for several reasons. First, if the E7 constructs are not easily crystallized in pRB-E7 complexes, pRB-E1A complexes could be produced for crystallization trials. Secondly, similar to HPV E7, the CR3 region of adenovirus E1A may also contain a second pRB binding site that allows for tighter pRB binding. In support of this hypothesis, Grand *et al.* demonstrated that the Adenovirus 12 E1A 13S gene product (an E1A protein which includes the

CR3 region) binds to a GST-pRB fusion protein with a higher affinity than the Adenovirus 12 E1A 12S gene product (an E1A protein without the CR3 region)[34]. The dissociation constant for E1A 12S-pRB complex was estimated to be higher than the dissociation constant E1A 13S-pRB complex by nearly two-fold. Additionally, the CR1 region of Adenovirus E1A also contains a separate pRB binding site[35]. This region of adenovirus E1A is necessary for the displacement of E2F transcription factors from pRB[35, 36]. Although the E1A CR1 region contains some homology with E7, there is no evidence that the E7 CR1 is necessary for E2F displacement or pRB interaction. Therefore, the three dimensional structure E1A-pRB complexes is expected to provide additional insight into the principles of high affinity pRB-protein interaction.

The E1A constructs expressed as soluble proteins at 37°C. E1A(36-189) was purified by utilizing a combination of anion exchange (Q-sepharose), dye affinity chromatography (Reactive red 120 and Blue sepharose), and gel filtration on a Superdex-200 FPLC column. However, gel filtration indicated that the E1A(36-189) protein existed as several differently sized multimers or aggregates. Additionally, this protein appeared to be susceptible to partial degradation. E1A(114-189) was purified to homogeneity by utilizing a combination of anion exchange (Q-sepharose), hydrophobic interaction (phenyl sepharose), and gel filtration on a Superdex-75 FPLC column. This E1A protein eluted from the gel filtration column in one peak. Unlike E7, the elution point of this E1A construct was consistent with the monomer molecular weight (8.376 kDa). Dynamic light scattering studies also indicated that E1A(114-189) was monomeric and monodisperse at 2 mg/ml.

The retinoblastoma tumor suppressor protein (pRB) contains two domains that are required for minimal viral oncoprotein interaction (domain A and domain B, Figure 4). These two domains are referred to as the small pocket of pRB. Several pRB constructs containing these domains were subcloned into pRSETA for bacterial expression. The cDNA for full length pRB was obtained from Dr. Robert Ricciardi. A total of 11 pRB constructs were generated by PCR and ligated into the pRSETA bacterial expression vector. Six of the pRB constructs are listed in Figure 4. Construct 1 through construct 5 all contain amino-terminal 6xhistidine tags for efficient purification with a Ni^{2+} column. Construct 1 (amino acids 376-928) contains the most biologically relevant region of pRB that is often referred to as the large pocket. Ewen *et al.* has demonstrated that the large pocket of pRB is sufficient for cell cycle regulation and for the association with E2F transcription factors[12]. Construct 2 (amino acids 376-843) contains the small pocket plus a C-terminal region that may provide a second viral oncoprotein binding site [33]. Construct 3 (amino acids 376-792) contains the small pocket of pRB which includes the flexible linker region that separates domain A and domain B. Construct 4 (amino acids 376-792 , Δ579-621) contains the small pocket with a deletion of amino acids 579-621 in the linker region. Construct 5 (amino acids 376-562) contains a region of domain A that has been crystallized previously[37]. Construct 6 (amino acids 622-792) contains domain B.

All of the pRB constructs (Figure 4) were bacterially expressed in BL21(DE3) cells. All constructs, except for construct 6, produced soluble proteins when induced at 15°C overnight. Construct 6 produced insoluble protein at both 37°C and 15°C. The soluble 6xhistidine tagged pRB proteins(from constructs 1-4) were purified by utilizing a combination of affinity chromatography (Ni-NTA agarose) and gel filtration on a Superdex-200 FPLC column. Each of these pRB proteins eluted from the gel filtration column in one peak consistent with the molecular weight of monomeric protein. Circular dichroism (CD) of pRB (376-792) and pRB(376-792 , Δ579-621) indicated that these proteins were respectively 55% and 60% helical. Further characterization of concentrated pRB proteins (1-2 mg/ml) by dynamic light scattering (DLS) indicated that pRB (376-792) and pRB(376-792 , Δ579-621) were monomeric at 4°C but aggregated with increasing temperature. pRB(376-928) appeared to be less stable than the other pRB-derived proteins because it precipitated at room temperature and DLS indicated that this protein was aggregated at 4°C at concentrations of 1-2mg/ml.

Initially, purified pRB proteins were mixed with purified HPV16 E7(1-98) protein in order to make pRB-E7 complexes. Since the stoichiometry of binding was unknown, pRB proteins were mixed with E7(1-98) at molar ratios of 1:1, 1:2, and 1:4. When these proteins were mixed at molar ratios 1:1 and 1:2, instant precipitation would occur. The precipitate contained both pRB and E7(1-98). When pRB proteins were mixed with E7(1-98) at a molar ratio of 1:4, precipitation did not occur. However, the existence of multiple broadened peaks on the gel filtration (Superdex-200) chromatogram for this complex indicated that there was aggregation of the complex as well as excess E7(1-98) that was not associated with pRB.

Since simply mixing the purified pRB proteins with purified E7(1-98) did not yield a stable complex, a bacterial coexpression system was utilized for these proteins. The rationale behind this was that perhaps these proteins were not in the right conformation for interaction when expressed and purified individually. However, if pRB was exposed to E7 during the protein expression and folding process, perhaps these proteins would adopt the correct conformation for interaction. Therefore, coexpressing pRB with E7 may promote the optimal situation for pRB-E7 complex formation. In order to coexpress the pRB proteins with E7, two pRB constructs were transferred into a slightly modified version of the pMR102 expression vector [38]. This T7-based expression vector contains a gene for kanamycin resistance and a M13 origin of replication which allows for compatibility with the pRSET A ampicillin resistant vector. The two pRB constructs used for this study were construct 3 (amino acids 376-792, Figure 4) and construct 4 (amino acids 376-792 , Δ579-621, Figure 4). These pRB constructs were initially chosen for coexpression because they encoded for proteins that were monodisperse and highly helical.

A pMR102 vector containing either the pRB(376-792) construct or the pRB(376-792 , Δ579-621) construct was cotransformed into BL21(DE3) with a pRSET vector containing one of the HPV16 E7 or E1A constructs. These constructs were tested for coexpression and solubility at 37°C and 15°C. An example of pRB(376-792) and HPV16 E7(1-98) coexpression is demonstrated in Figure 5. The coexpressed pRB(376-792) construct consistently yielded more protein than the pRB(376-792 , Δ579-621) construct during preliminary characterization. Therefore, only the pRB(376-792) construct was utilized for further characterization during these studies.

After pRB(376-792) was coexpressed at 15°C with either HPV16 E7 or E1A proteins, all proteins appeared to be soluble in low salt buffer. The 6xhistidine tag on pRB(376-792) was utilized during the first purification step. The soluble portion of the cell lysate was loaded onto a Ni-NTA agarose column followed by a wash with low salt buffer. The Ni^{2+} bound proteins were then eluted off of the Ni-NTA agarose column with imidazole. During the imidazole gradient, the viral oncoproteins coeluted with the 6xhistidine -tagged pRB(376-792). Fractions containing pRB and viral oncoproteins were then pooled and concentrated for further purification by gel filtration. Figure 6 demonstrates that the coexpressed pRB(376-792) and HPV16 E7(17-98) proteins coeluted from a Superdex-200 FPLC column. Coexpressed pRB(376-792) and HPV16 E7(17-98) proteins were assumed to exist in a complex because the coexpressed proteins coeluted from the gel filtration column at an estimated molecular weight that was higher than the individually purified components (Figure 7). A comparison to gel filtration protein standards indicated that the size of this pRB(376-792)-HPV16 E7(17-98) complex was approximately 158 kDa. This suggested that the molar ratio of pRB(376-792)-HPV16 E7(17-98) interaction was greater than 1:1. The coexpressed pRB(376-792)-E1A(36-189) and the coexpressed pRB(376-792)-E1A(114-189) constructs also coeluted during gel filtration on a Superdex 200-FPLC column (Figure 8). These purified complexes eluted from the gel filtration column at an estimated molecular weight that was higher than the molecular weight of pRB(376-792) and of E1A(114-189) alone. Unlike the pRB(376-792)-HPV16 E7(17-98) complex, these pRB-E1A complexes coeluted from the gel filtration column at molecular weights that are consistent with a stoichiometry of 1:1(Figure 8b). In agreement with this estimation, sedimentation equilibrium data of the pRB(376-792)-E1A(36-189)

complex indicated that the molecular weight of this complex was approximately 68 kilodaltons (sedimentation equilibrium data of pRB alone indicates that pRB(376-792) is a monomer of approximately 50 kDa). It is interesting to note that E1A(36-189) coeluted from the gel filtration column in one peak when it was bound to pRB(376-792) (Figure 8b). When E1A(36-189) was purified alone, it eluted from the column as several differently sized multimers or aggregates. This difference in aggregation state of E1A(36-189) suggests that there is strong interaction between pRB(376-792) and E1A(36-189). In support of this hypothesis, no dissociation of this complex was detected during analytical ultracentrifugation.

The coexpressed pRB(376-792)-HPV16 E7(17-98) complex and the pRB(376-792)-E1A complexes have been utilized for crystallization trials with several different factorial screens [39, 40] at 4°C. To date, these complexes have resisted crystallization. The recently determined three dimensional crystal structure of the pRB small pocket bound to a 9 amino acid E7 peptide has provided some insight into why these coexpressed complexes have not crystallized[31]. The crystallized pRB-E7 peptide complex was only able to crystallize when the linker region between domain A and domain B of pRB was removed by proteolysis[31]. The pRB(376-792) region that was used for coexpression and crystallization trials in this study included this flexible linker region. Therefore, the coexpressed pRB-viral oncoprotein complexes used in this study may have resisted crystallization because the pRB derivative contained this linker region.

Others have demonstrated that the flexible linker region is susceptible to proteolysis while the remainder of the pRB small pocket remains in tact[31, 41]. The pRB protein without the linker region is relevant for these studies because the flexible linker region is unnecessary for viral oncoprotein interaction[12]. In this study, preliminary small scale proteolysis studies of purified pRB(376-792) with trypsin indicate that pRB has two domains that are resistant to proteolysis [Figure 9a]. The molecular weights of the resulting peptides were consistent with the molecular weights of domain A and domain B. This suggests that the bacterially expressed pRB(376-792) construct has the same structural characteristics as the crystallized pRB region. An example of small scale proteolysis of the pRB(376-792)-HPV16 E7(17-98) complex is in Figure 9b. Small scale proteolysis studies of the pRB(376-792)-HPV16 E7(17-98) and the pRB(376-792)-E1A(114-189) complex resulted in the two pRB domains plus another protein that consistent with full length HPV16 E7(17-98) or E1A(114-189). Therefore, a protocol is currently being developed to perform large scale limited proteolysis of the purified pRB-viral oncoprotein complexes. These partially proteolyzed complexes will be characterized by gel filtration and utilized for crystallization trials. These experiments will also be performed with coexpressed pRB(376-843)-viral oncoprotein complexes. pRB(376-843) will be used because it includes a region C-terminal to the small pocket that has been shown to interact with the CR3 region of HPV16 E7[33]. This C-terminal region has been shown to be susceptible to limited proteolysis in bacterially expressed pRB[41]. Therefore, if this region is an E7 binding site, it is expected that this region would be protected by E7 during limited proteolysis of the pRB(376-843)-E7 complex. If this proves to be the case, partially proteolized pRB(376-843)-viral oncoprotein complexes will be utilized for crystallization trials as well.

The stage of research for this study is well within the expectations outlined in the Statement of Work section of the proposal. The second technical objective of the proposal was to prepare pRB-viral oncoprotein complexes that are suitable for X-ray crystallographic studies. As outlined in the proposal, the second technical objective is expected to be accomplished during months 5-18. At month 12, pRB-viral oncoprotein complexes have been prepared and isolated for crystallographic studies. It is expected that limited proteolysis of the coexpressed pRB-viral oncoprotein complexes will produce crystallizable protein within the next six months.

CONCLUSIONS:

It is important to disrupt pRB-cyclin D1 interaction in cyclin D1-mediated breast cancer. In this study, a structure-based approach is used to understand the molecular mechanisms that are necessary for high affinity pRB-viral oncoprotein interaction. Since viral oncoproteins are expected to compete with and possibly imitate some interactions that pRB normally has with cyclin D1, this information will provide tremendous insight into the specific interactions required for the development of small compounds that can destabilize pRB-cyclin D1 complexes in cyclin D1-mediated breast cancer. The recently determined pRB-E7 peptide crystal structure has provided insight into pRB-LXCXE interaction[31]. However, there are some significant shortcomings to this structure because the crystallized E7 nine amino acid peptide binds to pRB with a twenty-fold weaker affinity compared to full-length HPV16 E7[32]. In order to address these shortcomings, this study proposes to elucidate the molecular mechanisms of high affinity pRB-viral oncoprotein interaction. A region that is C-terminal to the LXCXE region of E7 and of E1A makes additional contacts with pRB[33, 34]. Furthermore, the E1A CR1 region has been shown to interact with pRB and is necessary for the displacement of E2F transcription factors[36]. Therefore, the primary goal of this project is to determine the three dimensional structure of pRB bound to the larger constructs of HPV16 E7 , HPV1a E7, and Adenovirus 5 E1A utilized in this study.

This study demonstrated that bacterially expressed pRB is capable of interacting with bacterially expressed HPV16 E7 and Adenovirus5 E1A derivatives that contain biologically relevant high affinity pRB binding regions. Each of the bacterially coexpressed pRB(376-792)-HPV16 E7(17-98), pRB(376-792)-E1A(36-189), and pRB(376-792)-E1A(114-189) proteins formed complexes. Each of these complexes eluted from a gel filtration column as one peak consistent with a molecular weight that was higher than the individually purified components. The estimated molecular weight of the pRB(376-792)-HPV16 E7(17-98) complex by gel filtration suggested that the molar ratio of this complex was greater than 1:1. The coexpressed pRB(376-792)-E1A complexes eluted from the gel filtration column at molecular weights that were consistent with a stoichiometry of 1:1. In support of this, analytical ultracentrifugation of the pRB(376-792)-E1A(36-189) complex suggested that this complex existed as a single species of 68kDa (the estimated molecular weight of a 1:1 complex). This data is also consistent with the observed stoichiometry of binding in the pRB-E7 peptide crystal structure[31].

Based on the linkerless crystallized pRB region, the purified coexpressed pRB(376-792)-viral oncoprotein complexes probably have resisted crystallization because this pRB derivative included the flexible linker region between domain A and domain B[31, 41]. Preliminary data suggests that partial digestion of the coexpressed pRB(376-792)-HPV16 E7(17-98) complex or the coexpressed pRB(376-792)-E1A(114-189) complex with trypsin yield three protein fragments that are consistent with the molecular weights of domain A and domain B (of pRB) as well as full length E7(17-98) [Figure 9b] or E1A(114-189). Therefore, limited proteolysis will be utilized to modify these complexes for future crystallization trials. The stage of research for this study is well within the expectations outlined in the Statement of Work section of the proposal.

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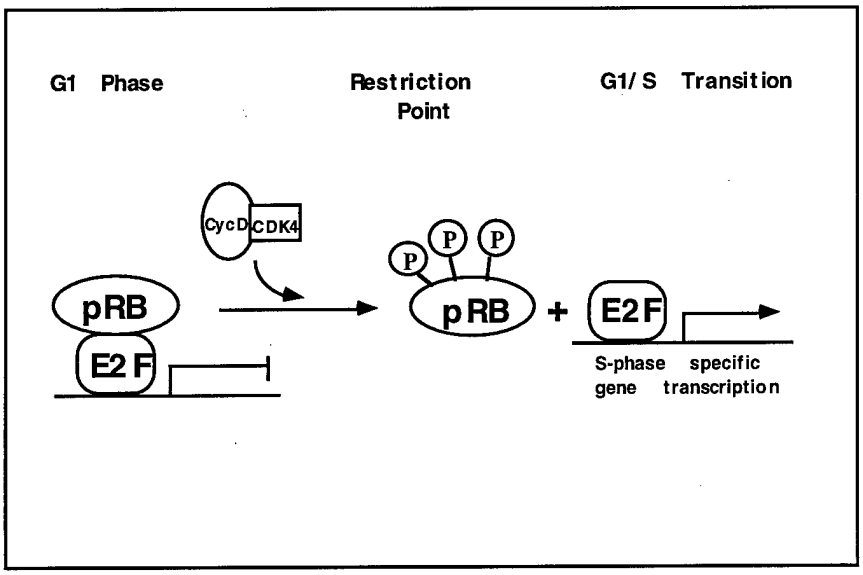


Figure 1: Schematic Representation of pRB Inactivation. In G1 phase, pRB binds to and inactivates E2F transcription factors. During the restriction point, pRB is phosphorylated by cyclinD/cyclin-dependent kinase 4 (CDK4) complexes. This phosphorylation causes pRB to release E2F transcription factors. These transcription factors are then able to stimulate the transcription of S-phase specific genes that are involved in cellular proliferation.

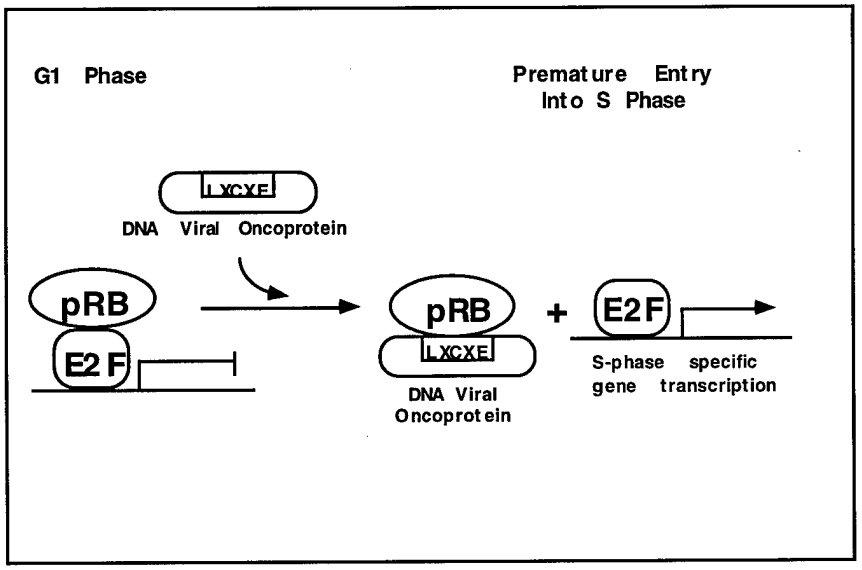


Figure 2: Schematic Representation of Premature pRB Inactivation by DNA Viral Oncoproteins. DNA viral oncoproteins adenovirus E1A, SV40 Large T antigen, and HPV16 E7 bind to hypophosphorylated pRB. The conserved sequence on these viral oncoproteins that is necessary for this interaction contains amino acids LXCXE (where X is any amino acid). Additionally, Cyclin D and other cellular proteins utilize this sequence to interact with pRB. The DNA viral oncoproteins displace E2F transcription factors from pRB, which leads to premature transcripton of S-phase specific genes and early progression into S phase.

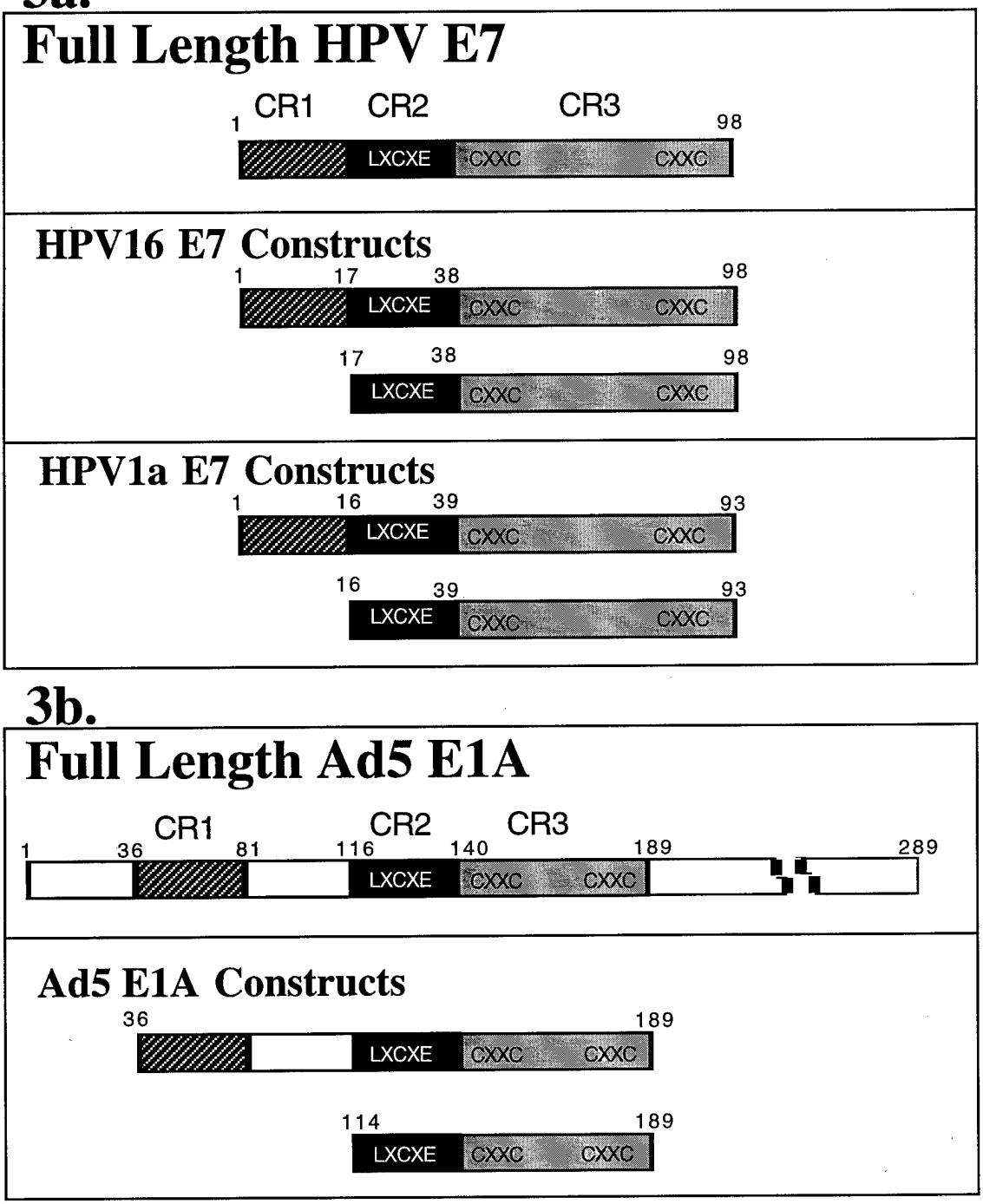


Figure 3: Schematic Representation of Bacterially Expressed HPV E7 and Adenovirus 5 E1A Constructs. These viral oncoproteins contain three conserved regions (CR1, CR2, CR3). CR2 contains the minimal pRB binding region LXCXE (where X represents any amino acid). CR3 contains a Zn2+ binding region which consists of two CXXC motifs separated by a linker. CR3 is expected to have a separate pRB binding site. **3a.** Two HPV16 E7 constructs encode for full length HPV16 E7(1-98) (contains CR1-CR3) and for HPV16 E7(17-98) (contains CR2-CR3). Similar HPV1a E7 constructs were generated that encode for HPV1a E7(1-93) and HPV1a E7(16-93). **3b.** Comparable Adenovirus 5 E1A constructs containing CR1-CR3 (amino acids 36-189) and CR2-CR3 (amino acids 114-189) were also generated. All constructs were subcloned into pRSET A for bacterial expression.

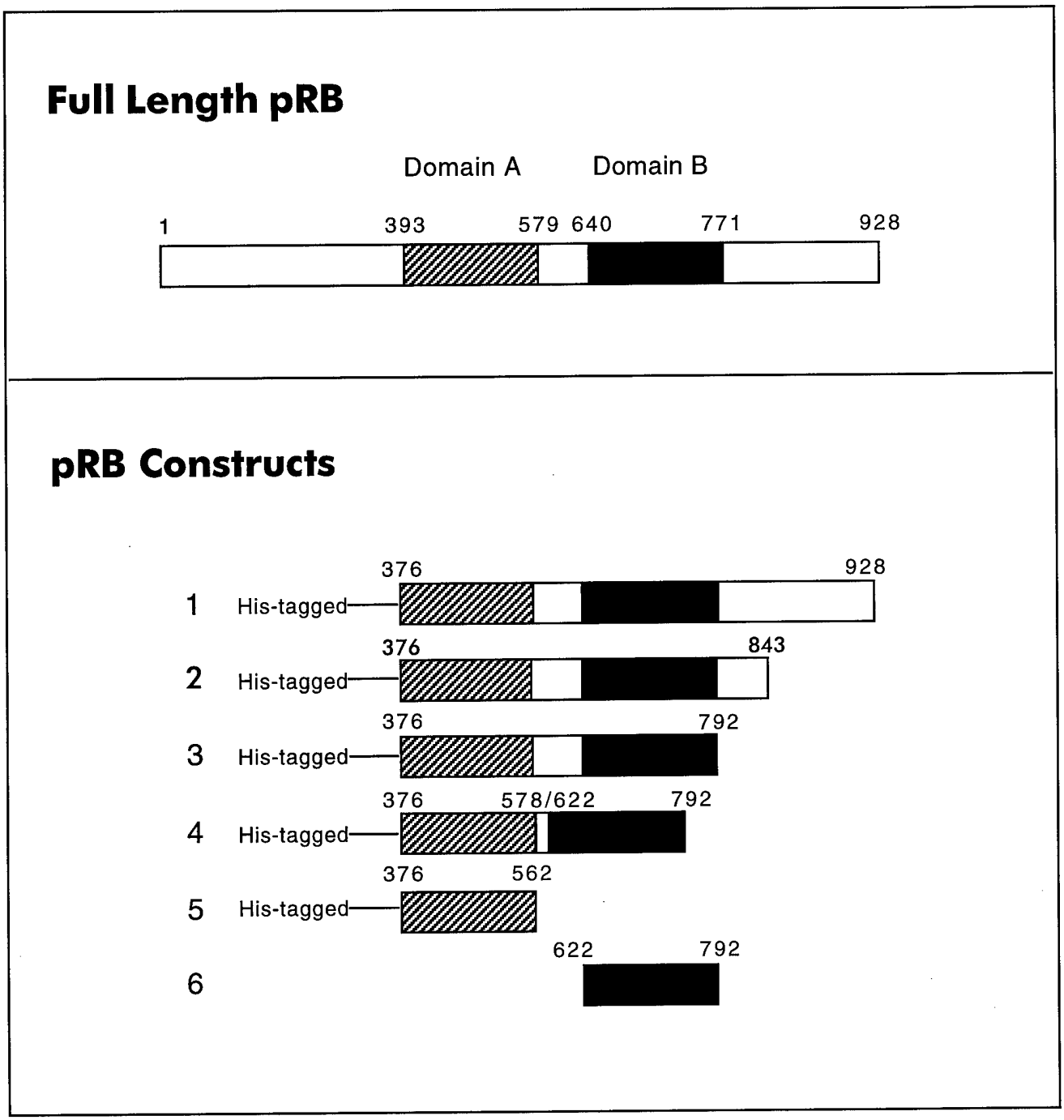


Figure 4: Schematic Representation of Bacterially Expressed pRB Constructs. pRB is a 928 amino acid protein that contains two domains which are necessary for viral oncoprotein interaction. These domains are referred to as Domain A and Domain B. These domains are separated by a linker region that is unnecessary for viral oncoprotein interaction. Six pRB constructs are shown. Construct 1 contains amino acids 376-928. Construct 2 contains amino acids 376-843. Construct 3 contains amino acids 376-792. Construct 4 contains amino acids 376-792 with a deletion of amino acids 579-621. Constructs 1-4 contain both Domain A and Domain B. Construct 5 contains amino acids 376-562. (Domain A) Construct 6 contains amino acids 622-792 (Domain B). Construct 1 through construct 5 all contain amino-terminal 6x histidine tags for efficient purification with a Ni2+ column.

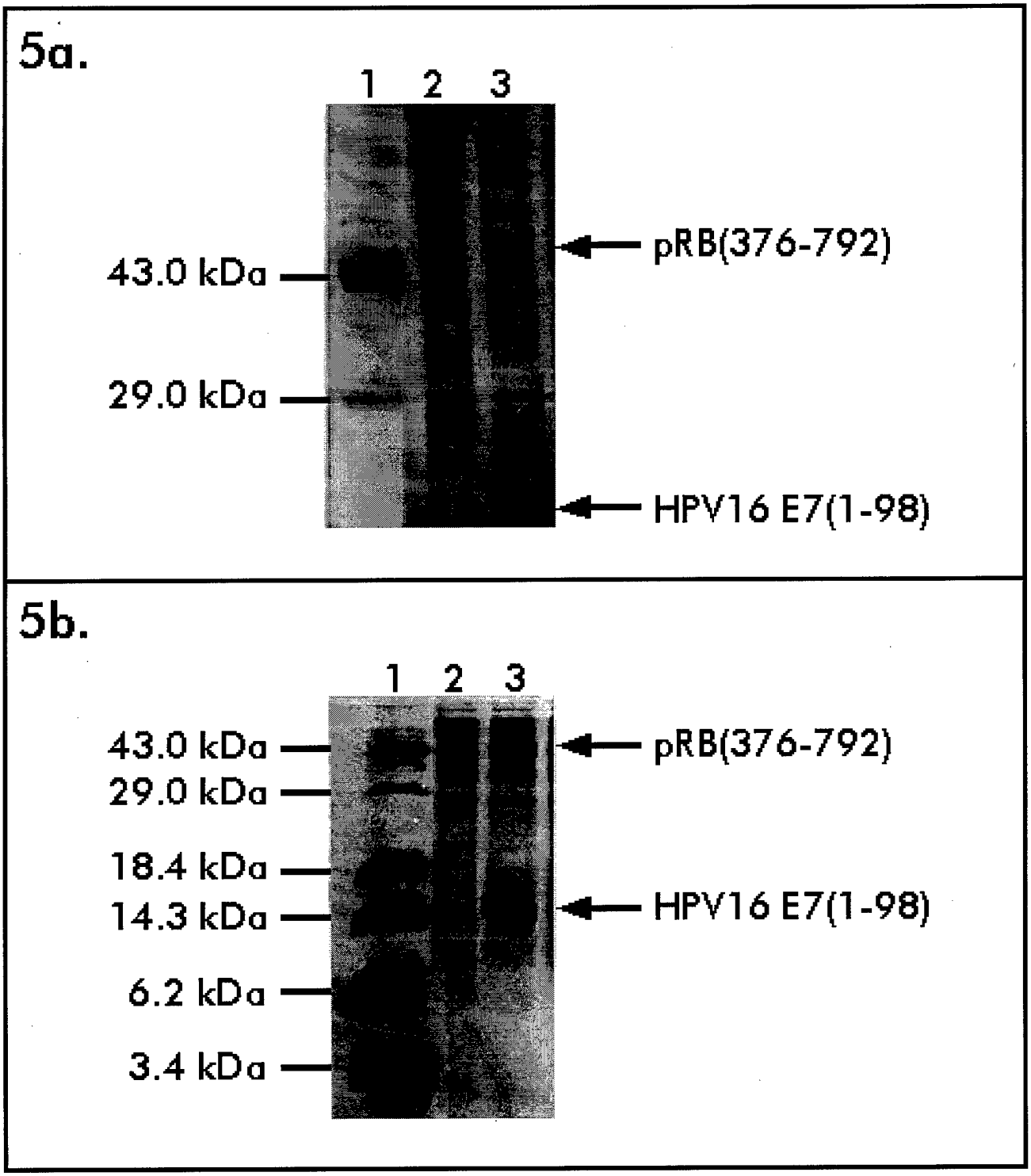
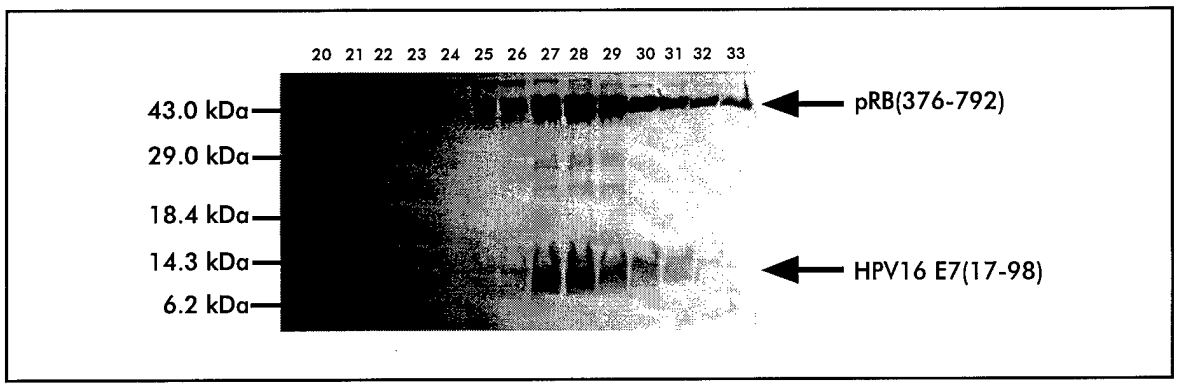


Figure 5: pRB(376-792) is Coexpressed with HPV16 E7(1-98) in Bacteria. 5a. A 15% SDS-polyacrylamide gel contains molecular weight standards (lane 1), uninduced cotransformed BL21(DE3) cells (lane 2), and induced BL21(DE3) cells that were cotransformed with HPV16 E7 (1-98) and pRB(376-792) (lane 3). This gel was optimal for resolving induced pRB(376-792) protein. 5b. The same samples were loaded onto a 22% SDS-polyacrylamide gel. This gel contains molecular weight standards (lane 1), uninduced cotransformed BL21(DE3) cells (lane 2), and induced BL21(DE3) cells that were cotransformed with HPV16 E7 (1-98) and pRB(376-792) (lane 3). This gel was optimal for resolving the induced HPV16 E7(1-98) protein.

6a.



6b.

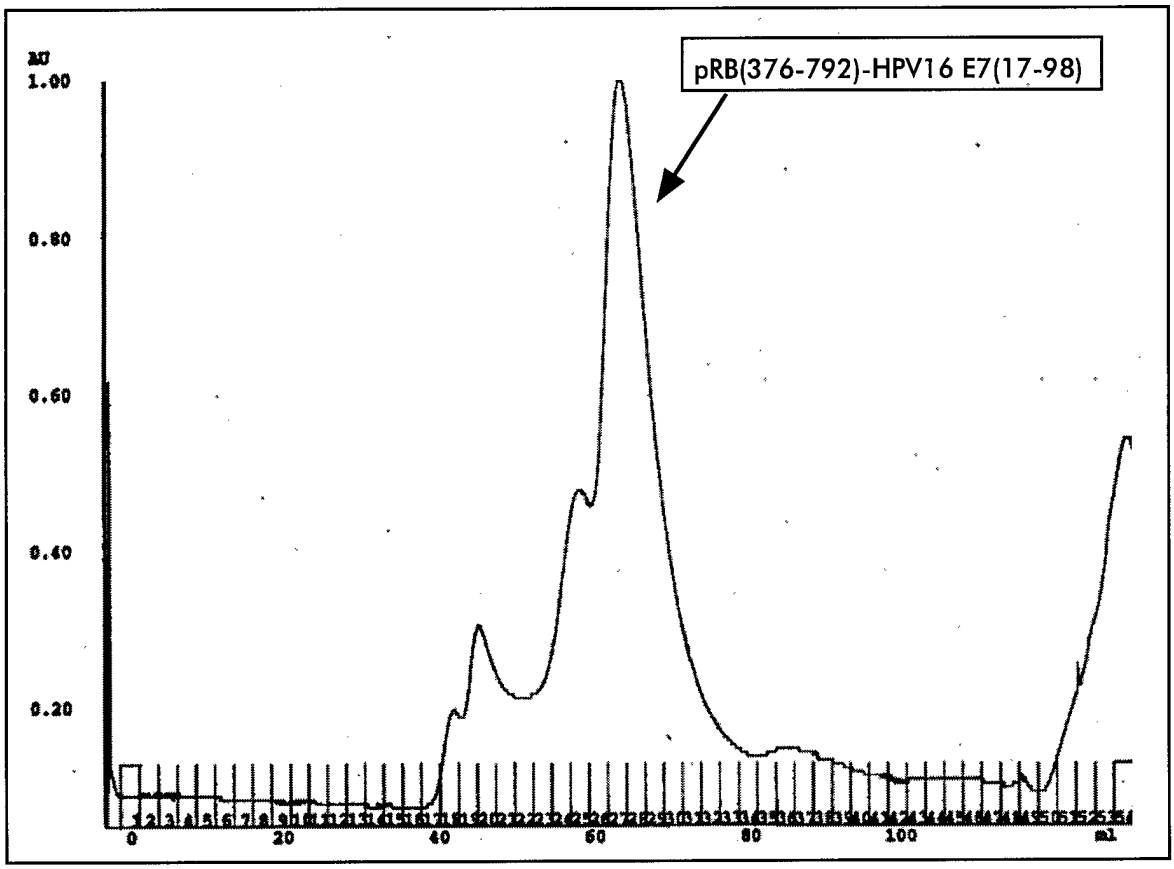


Figure 6: Coexpressed pRB(376-792) and HPV16 E7(17-98) Coelute from a Gel Filtration Column. 6a. A 22% SDS-polyacrylamide gel contains molecular weight standards and fractions 20-33 from size exclusion chromatography using a Superdex-200 FPLC column. 6b. The major peak in the gel filtration chromatogram corresponds with the coelution of pRB(376-792) and HPV16 E7(17-98). A comparison to gel filtration protein standards indicate that the coelution point of the pRB(376-792) and HPV16 E7(17-98) proteins corresponds to a molecular weight of 158 kilodaltons. The buffer used in this column contained 20mM Hepes, pH 7.5, 200mM NaCl, and 20mM DTT.

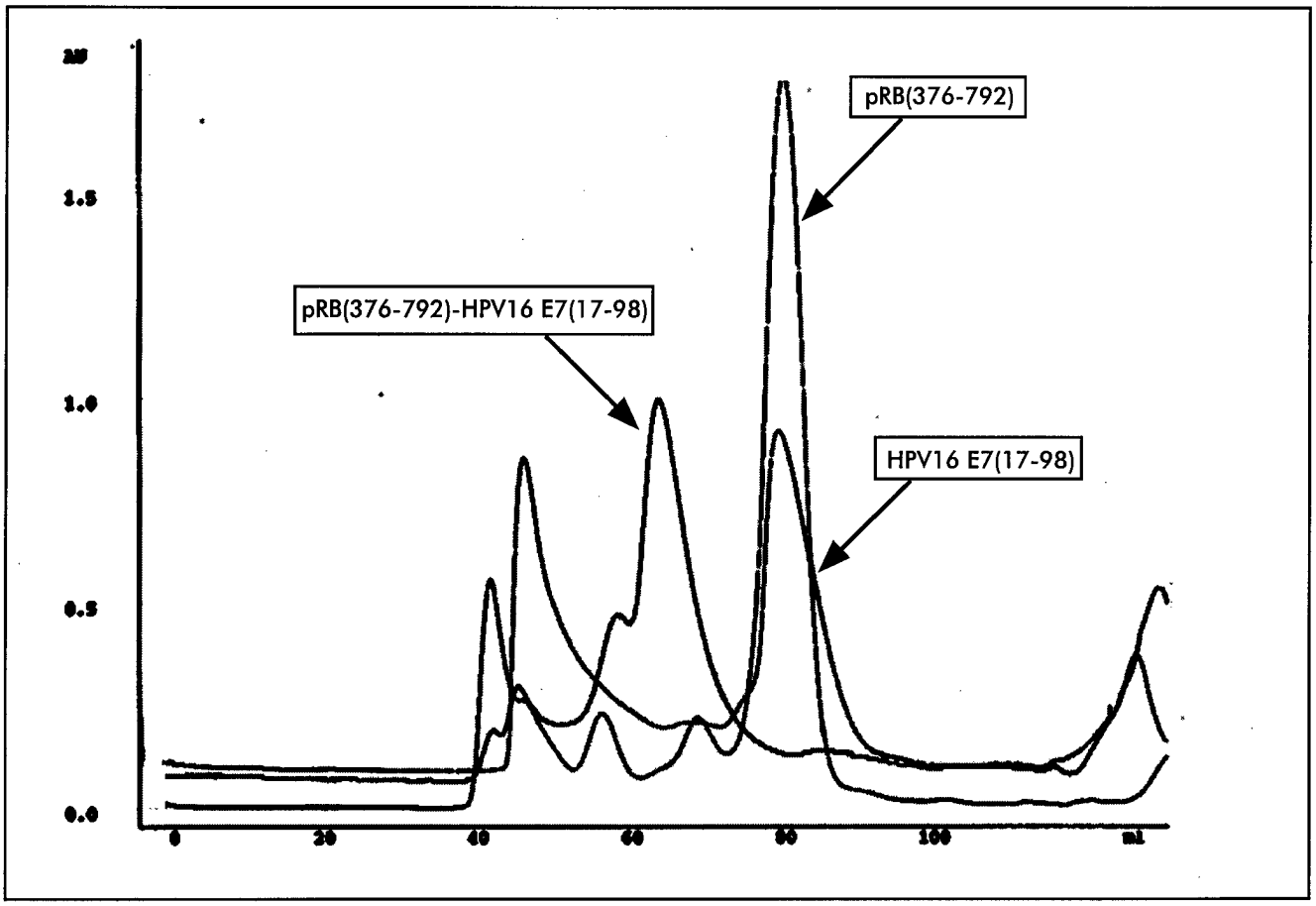
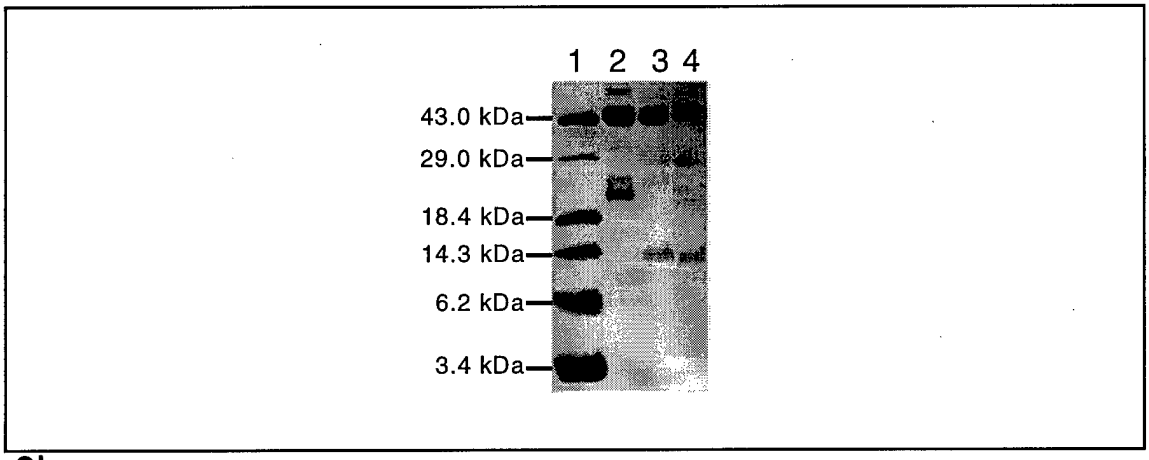


Figure 7: Coexpressed pRB(376-792) and HPV16 E7(17-98) Coelute from a Gel Filtration Column Earlier than Individually Purified pRB(376-792) or HPV16 E7(17-98). The Superdex 200 gel filtration chromatogram of coexpressed pRB(376-792)-HPV16 E7(17-98) is overlaid with the Superdex 200 gel filtration chromatograms of individually purified pRB(376-792) and individually purified HPV16 E7(17-98). A comparison of the chromatograms demonstrate that coexpressed pRB(376-792)-HPV16 E7(17-98) proteins coelute from the column as a larger molecular weight species than the individual components

8a.



8b.

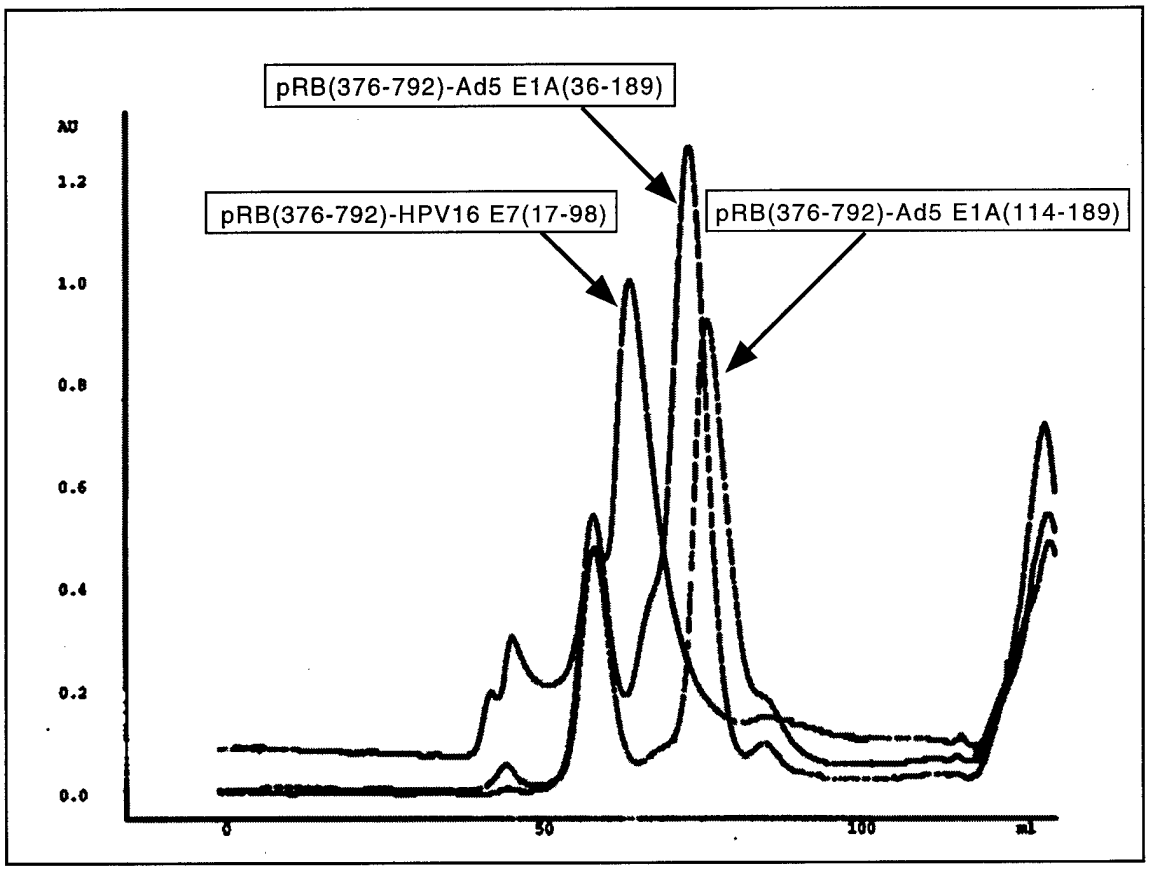
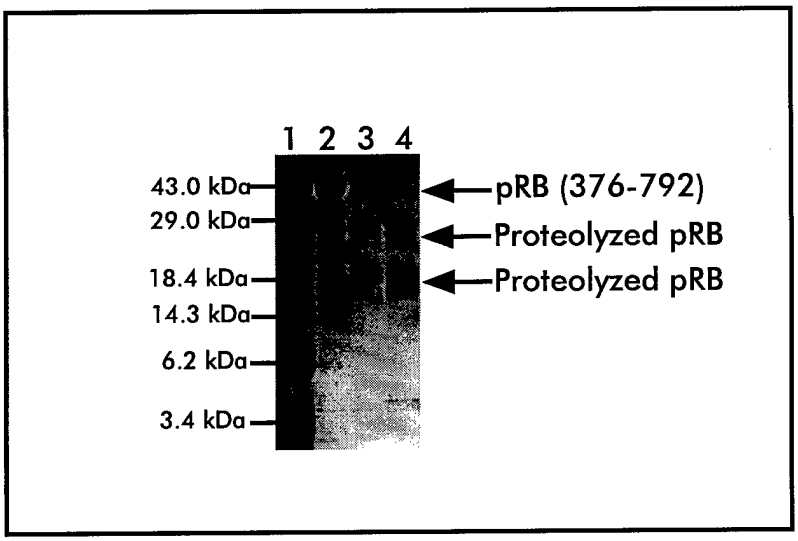


Figure 8: Coexpressed pRB(376-792)-HPV16 E7(17-98), pRB(376-792)-E1A(36-189), and pRB(376-792)-E1A(114-189) are Purified Using Gel Filtration. 8a. A 22% SDS-polyacrylamide gel contains molecular weight standards (lane 1), purified pRB(376-792)-E1A(36-189) (lane 2), purified pRB(376-792)-E1A(114-189) (lane 3), and purified pRB(376-792)-HPV16 E7(17-98) (lane 4). pRB migrates near the 43 kilodalton standard in lanes 2-4. The viral oncoproteins are the lower molecular weight proteins in lanes 2-4. 8b. The Superdex 200 gel filtration chromatogram of coexpressed pRB(376-792)-HPV16 E7(17-98) is overlaid with the Superdex 200 gel filtration chromatograms of coexpressed pRB(376-792)-E1A proteins. A comparison of the chromatograms demonstrate that coexpressed pRB(376-792)-HPV16 E7(17-98) proteins coelute from the column as a larger molecular weight species than the pRB(376-792)-E1A complexes. All gel filtration chromatography was performed in a buffer containing 20mM Hepes, pH 7.5, 200mM NaCl, and 20mM DTT.

9a.



9b.

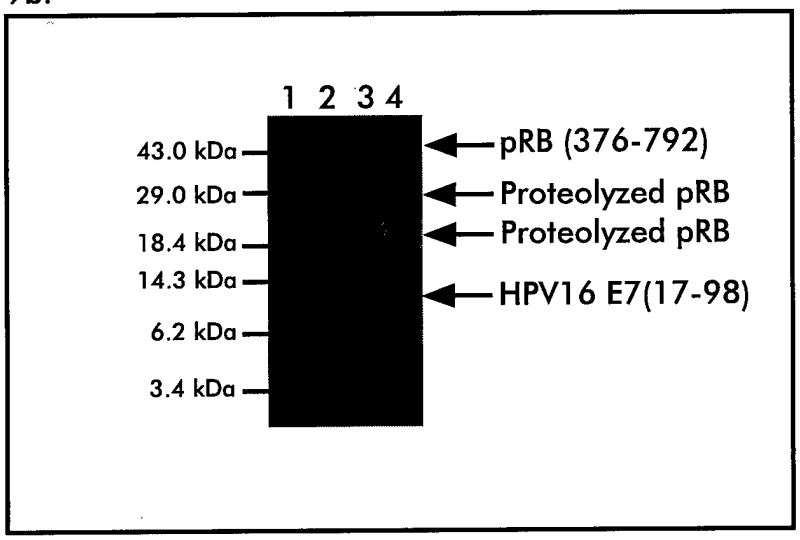


Figure 9: Small Scale Trypsin Digests of pRB(376-792) and Coexpressed pRB(376-792)-HPV16 E7(17-98) Complex. 9a. A 22% SDS-Polyacrylamide gel demonstrates that proteolysis of pRB(376-792) with trypsin yields two major protein fragments that are resistant to proteolysis. The molecular weights of these protein bands are consistent with the molecular weights of the crystallized pRB Domains A and B[31] (25.0kDa and 18.3kDa respectively). Lane 1 contains molecular weight standards. Lane 2 contains 3ug of undigested pRB(376-792)(the purified pRB protein was several months old which may explain the partial degradation of pRB). Lane 3 contains 3ug of pRB(376-392) that was digested with 0.025ug of Trypsin. Lane 4 contains 3ug of pRB(376-392) that was digested with 0.25ug of Trypsin. 9b. A 22% SDS-Polyacrylamide gel demonstrates that trypsin proteolysis of the coexpressed pRB(376-792)-HPV16 E7(17-98) complex yields three major protein fragments that are resistant to proteolysis. The molecular weights of these protein bands are consistent with the molecular weights of the crystallized pRB Domains A and B[31] and full length HPV16 E7(17-98). Lane 1 contains molecular weight standards. Lane 2 contains 5ug of undigested coexpressed pRB(376-792)-HPV16 E7(17-98) complex. Lane 3 contains 5ug of coexpressed pRB(376-792)-HPV16 E7(17-98) complex with 0.025ug of trypsin. Lane 4 contains 5ug of coexpressed pRB(376-792)-HPV16 E7(17-98) complex with 0.25ug of trypsin. All reactions took place in 10ul at 4oC for 20 minutes. The reaction was stopped by the addition of SDS-PAGE loading dye.